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Association of Neurotrophin Receptor Expression and Differentiation in Human Neuroblastoma

Jeff C. Hoehner,*† Leif Olsen,† Bengt Sandstedt,‡ David R. Kaplan,§ and Sven Påhlman*

From the Department of Pathology and the Department of Pediatric Surgery,† Uppsala University Hospital, Uppsala, Sweden; the Department of Pediatric Pathology,‡ Karolinska Hospital, Stockholm, Sweden; and ABL-Basic Research Program,§ National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland*

Interactions of the* trk *family of tyrosine kinase receptors with neurotrophins result in growth and maturational changes in neuronal cells. The continued progression, maturation, or regression of neuroblastoma, an embryonal, sympathetic nervous system-derived tumor of infants and children, might be governed by neurotrophic influences. Immunocytochemistry was utilized to evaluate TrkA, TrkB, and TrkC protein expression at the cellular level in the developing human fetal sympathetic nervous system and in a selection of neuroblastoma tumor specimens. TrkA and TrkC expression was identified in sympathetic ganglia and within the adrenal medulla, with intense TrkB expression restricted to paraganglia, of the normal developing human sympathetic nervous system. In neuroblastoma, pp140^{trkA} expression correlated positively with favorable tumor stage (P = 0.0027) and favorable outcome (P = 0.026). No statistically significant correlation of TrkC expression with outcome was evident; however, both TrkA and TrkC expression was most apparent in tumor cells of increased differentiation. TrkB expression was primarily localized to cells within the fibrovascular tumor stroma. A model of neurotrophin receptor expression and neurotrophin reactivity with differentiation is proposed. The existence and spatial distribution of neurotrophin receptors in neuroblastoma lend supportive evidence that neurotrophic influences may be involved in tumor persistence or regression. (Am J Pathol 1995, 147:102–113)

Nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin (NT)-3, and NT-4/5, the neurotrophin family of growth factors, promote differentiation, growth, and survival of central and peripheral nervous system neurons.[1–3] All members are synthesized as precursor polypeptides that, through enzymatic cleavage, yield mature homodimeric neurotrophins that differ in their sites of developmental expression and neuronal targets.[3] Neurotrophic interactions with receptors of target neurons result in a variety of trophic and tropic events. NGF is recognized to promote survival and differentiation of neural crest sensory and sympathetic neurons among others.[3] Brain-derived neurotrophic factor supports survival and outgrowth of a variety of central and neural crest-derived neurons but not sympathetic neurons. Both NT-3 and NT-4/5 have been described to act in a variety of neuronal locations.

The tyrosine kinase family of receptors encoded by the *trk* proto-oncogenes has been shown to be an essential component for neurotrophin interaction with neuronal cells.[4–8] TrkA is the high affinity receptor for NGF, TrkB is the receptor for brain-derived neurotrophic factor and TrkC the receptor for NT-3. Some crossover exists as both TrkA and TrkB bind NT-3 and NT-4/5; however, they transduce their signals with lesser efficacy. Examination of the developing nervous system has confirmed the developmental regulation of the Trk receptors.[9–13]

Neuroblastoma (NB) is an embryonic, sympathetic nervous system-derived tumor of infancy and childhood, which may arise at any sympathetic nervous tissue location. Prognostic variability partly depends upon patient age at diagnosis and tumor stage, younger patients with less tumor burden enjoying the best prognosis.[14–16] One intriguing feature of NB is

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Address reprint requests to Dr. Sven Påhlman, Department of Pathology, Uppsala University Hospital, S-75185 Uppsala, Sweden.

that some disseminated tumors in infants may regress spontaneously even without treatment.[17,18] Numerous investigations have sought tumor markers that might predict survival and guide therapy. N-*myc* amplification, degree of neuronal differentiation, mitosis-karyorrhexis index, *src* splicing variants, and levels of catecholamine metabolites are predictive of patient outcome.[14,15,19–22] Recently, expression of insulin-like growth factor II mRNA and extent of cellular apoptosis have been shown to correlate inversely with disease aggressiveness; apoptosis is most apparent in tumor regions with increased cellular differentiation.[23,24] Alterations in expression of the low affinity NGF receptor, $p75^{NGFR}$, have also been reported in clinical and cell culture NB materials.[20,25] Similarly, low affinity NGF receptor and *trkA* mRNA expression have been shown to correlate with improved outcome in clinical tumors.[20,25–27] In two cases in which primary NB cultures expressed *trkA*, NGF induced terminal differentiation whereas NGF deprivation resulted in cell death.[20]

Because of the prognostic heterogeneity of NB with respect to stage of disease, apoptosis, and neurotrophin-directed maturation, we wished to further investigate high affinity neurotrophin receptor protein expression at the cellular level in NB and within the normal developing human sympathetic nervous system. We postulate that neurotrophin influences are involved in NB tumor cell maturation and predict that a loss of neurotrophin support could result in tumor cell death via apoptosis.

Materials and Methods

Tumor and Fetal Materials

Pertinent clinical features and materials were obtained from 36 patients registered and treated for NB (28), ganglioneuroblastoma (4), or ganglioneuroma (4) in Sweden over the past 7 years. Pathological materials from biopsy or resection of primary or metastatic tumor were evaluated and the diagnosis was confirmed at one of four referral hospitals in Göteborg, Lund, Stockholm, or Uppsala, Sweden. Because of the relative scarcity of low stage tumors, a representative selection of high stage tumors as well as all low stage tumors obtainable were studied. Tumor stage was determined clinically or at the time of surgical biopsy or resection according to the criteria of Evans et al.[16] Patient characteristics were obtained from the treating clinicians. At a median follow-up of 31 months, all 10 patients diagnosed with stage I or stage II disease were alive and free of disease. Of the 10 patients with stage IV disease, 6 have died (60%), 1 patient remains alive with active disease, and 3 patients remain free of disease at a median follow-up of 29 months. N-*myc* amplification of greater than 10-fold was associated with 100% mortality (4/4). Patients were at varying stages of treatment at the time of tissue acquisition. The majority of patients with stage III and stage IV disease (60%) received cytotoxic therapy before the time of tumor biopsy or resection whereas those with stage I, II, and IVS had not (0%).

Human fetal tissue was obtained from both elective and spontaneous abortions from 9 to 25 weeks gestational age. Ethical approval (Dnr93–216) was obtained from the local ethical committee of Karolinska Hospital, Stockholm, Sweden.

Tissue Preparation and Immunocytochemistry

Tissue samples were fixed in 4% buffered formaldehyde and embedded in paraffin. Sections of 4 to 5 μm were secured to slides pretreated with silane and acetone and deparaffinized. Sections were subjected to microwave treatment in 10 mmol/L sodium citrate buffer, pH 7.3, for 5 minutes at 750 watts and 10 minutes at 450 watts. Sections were then cooled in deionized distilled water (DDW) and blocked with 0.1% bovine serum albumin (BSA) in Tris-HCl-buffered saline for 20 minutes. Primary antibody at optimal dilution was added to sections with a 60-minute incubation at room termperature. Slides were rinsed three times with Tris-buffered saline buffer and incubated for 30 minutes at room temperature with alkaline phosphatase-conjugated monoclonal mouse anti-rabbit antibody (Dakopatts, Glostrup, Denmark) at 1:40 dilution. Sections were again washed and developed with Fast-red TR salt as chromogen according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO). Slides were then rinsed in tap water for 10 minutes and counterstained with hematoxylin. Coverslips were applied in aqueous medium.

Antisera and Antisera Characteristics

Primary antibodies included polyclonal rabbit Trk (TrkA immunoglobulin (763); Santa Cruz Biotechnology, Santa Cruz, CA), epitope corresponding to amino acids 763 to 777 mapping adjacent to the carboxy terminus of human *trk* p140 (1:100 dilution); polyclonal rabbit TrkB immunoglobulin (794; Santa Cruz Biotechnology), epitope corresponding to amino acids 794 to 808 mapping adjacent to the carboxy terminus of human *trkB* p145 (1:100 dilution);

truncated polyclonal rabbit TrkB immunoglobulin (C-13; Santa Cruz Biotechnology), epitope corresponding to amino acids mapping at the carboxy terminus of human truncated *trkB* p95 (1:100 dilution); and polyclonal rabbit TrkC immunoglobulin (798; Santa Cruz Biotechnology), epitope corresponding to amino acids 798 to 812 mapping adjacent to the carboxy terminus of porcine *trkC* p140 (1:100 dilution). Additionally, anti-Trk antisera corresponding to both intracellular and extracellular domains of TrkA, TrkB, and TrkC proteins (TrkAin, TrkAout, TrkBin, TrkBout, TrkCin, TrkCout, and panTrk)[28–30] were investigated. Optimal antibody concentration as determined by serial dilutions was used in all instances. Equivalent immunoreactivity was obtained in paraffin-embedded and in similarly prepared snap-frozen sections of corresponding tumors. Negative controls were obtained by exclusion of primary antibody or by incubation with nonimmune bovine serum albumin at equivalent concentrations. Preincubation of the anti-TrkA, anti-TrkB, and anti-TrkC antisera with the corresponding peptides used for immunization (Santa Cruz Biotechnology) resulted in complete loss of specific immunoreactivity for respective antisera. Cross-incubation of these peptides to the antisera, eg, TrkC control peptide addition to anti-TrkA antisera incubations, did not affect the specific immunocytochemical staining.

To further characterize the antisera used, Western blot analyses of cell lysates from Sf9 insect cells infected with recombinant baculoviruses expressing TrkA,[31] TrkB (RM Stephens, D Soppet, L Parada, DR Kaplan, unpublished observations), or TrkC (P Tsoulfas, L Parada, unpublished observations) were performed. Cell lines were provided by Dr. Anna Maroney and Dr. Craig Dionne (Cephalon, West Chester, PA). Cell lysates were prepared as previously described,[32] electrophoresed on 7.5% sodium dodecyl sulfate polyacrylamide gels, transferred to nitrocellulose filters, probed with the above described Trk antibodies,[32] and developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Antisera specificities were also investigated by using NIH-3T3 cells specifically transfected with TrkA, TrkB, or TrkC, grown to semiconfluence, washed with phosphate-buffered saline (PBS), fixed for 10 minutes in methanol, and subjected to the immunocytochemical protocol as above.

Immunocytochemistry utilizing anti-neuron-specific enolase (NSE) IgG antiserum (Dakopatts) at 1:50 dilution, anti-S100 IgG antiserum (Dakopatts) at 1:100 dilution, and anti-chromogranin A&B IgG antiserum (Dakopatts) at 1:2000 dilution was performed in similar fashion.

DNA Nick End-Labeling of Tissue Sections

A procedure to specifically end label DNA cleavage sites in tissue sections *in situ* (TUNEL) was employed as previously described with minor modifications.[33] Paraffin-embedded sections were deparaffinized and incubated in a moist chamber for 15 minutes with 20 µg/ml proteinase K (Sigma Chemical Co.) and thereafter washed three times with DDW. Two percent hydrogen peroxide was added for 5 minutes to inactivate endogenous peroxidase, and the sections were again washed three times with DDW. Each section was then incubated at 37°C for 60 minutes with terminal deoxynucleotidyl transferase (10 enzymatic units/50 µl) and biotinylated deoxyuridine (dUTP, 0.5 nmol/50 µl; both Boehringer Mannheim, Mannheim, Germany) in transferase buffer (30 mmol/L Tris-HCl buffer, pH 7.2, 140 mmol/L sodium cacodylate, and 1 mmol/L cobalt chloride). Reaction was terminated by immersion in 300 mmol/L NaCl and 30 mmol/L sodium citrate buffer for 15 minutes. Sections were washed three times with DDW, blocked for 10 minutes with 2% bovine serum albumin at room temperature, and washed three times again with DDW. Sections were immersed in PBS for 5 minutes and then incubated with avidin-biotin complex (1:100 avidin and 1:100 biotinylated horseradish peroxidase in 0.1% bovine serum albumin; Dakopatts) for 30 minutes according to the manufacturer's instructions. Sections were washed three times with DDW, immersed in PBS for 5 minutes, developed in 3-amino-9-ethylcarbazole solution for 20 minutes at room temperature, and washed for 10 minutes in DDW. Coverslips were applied in aqueous medium.

Quantitation of Trk Expression

A semiquantitative scale to grade the degree of Trk immunoreactivity was used in all NB specimens. A scale from 0 to 5 was used: 0, no staining; 1, trace immunoreactivity evident; 2, occasional cells stain; 3, minority of cells stain; 4, majority of cells stain; and 5, all cells stain intensely. Fifteen randomly selected regions of each specimen were examined under high power, each region was rated, and the median rating of the fifteen regions was determined. Care was taken to avoid examining regions with apparent necrosis. Consistent results were obtained between repeat experiments and between different block sections of tumor tissue from the same patient. Standardized specimens were analyzed with each assay to ensure reproducibility of the scoring scale. Immunocytological examination was blinded with respect to patient outcome and tumor stage. The mean and standard deviation of immunocytochemical staining grade for

all tumors stratified according to tumor stage and outcome was calculated, and statistical comparison performed by Student's *t*-test.

Results

Anti-Trk Antisera Specificities

Three sets of anti-Trk antisera were used, one commercially available and the other two generated by independent research groups. As mentioned below, and exemplified in Figure 1, these antisera gave equivalent results in normal fetal tissue and in tumor sections but with somewhat increased nonspecific background reactivity for some antisera when staining tumor sections. Identical results were obtained comparing the differing antisera for a given protein in other neuron-harboring tissues, ie, sections of human fetal and postnatal intestine (JC Hoehner, T Wester, D Kaplan, S Påhlman, L Olsen, manuscript in preparation).

The similar immunocytochemical reactivity patterns resulting from anti-TrkA and anti-TrkC antisera prompted a more careful characterization of the specificities of these antisera to rule out cross-reactivity between anti-TrkC antisera and TrkA and vice versa. To that end, cell lysates from TrkA-, TrkB-, and TrkC-overexpressing Sf9 cells were analyzed by Western blotting with those antisera that perform well in Western protocols. The anti-TrkA (763) antisera, as well as all other specific, noncommercial antisera tested, recognized one major protein with the expected molecular weight, in the respective transfectant (Figure 2A and data not shown).

The antisera specificities were similarly tested in the immunohistochemical protocol with Trk-overexpressing NIH-3T3 cells. Immunoreactive positivity was identified only in transfected cells with the respective specific appropriate antisera. In particular, the anti-TrkC antiserum (798) did not stain the TrkA-overexpressing cells, and the anti-TrkA antiserum (763) did not stain the TrkC-transfected cells (Figure 2, B and C). Thus, no cross-reactivity of specific antibodies to differing Trk-overexpressing cells was identified in any of the three cell lines when tested with all above listed antisera. Furthermore, antisera directed to the same Trk species gave equivalent staining results both in normal and in tumor tissues; however, slight differences in background staining were evident, and the best results were obtained with the anti-TrkA (763) and anti-TrkB (794) antisera.

Neurotrophin Receptor Expression in Human Fetal Materials

Results of TrkA, TrkB, and TrkC immunoreactivities at human fetal gestational ages 9 to 24 weeks are summarized in Table 1. In general, immunoreactivity increased in tissues of the developing sympathetic nervous system with increasing gestational ages. Neuronal cells of dorsal root ganglia, pre-aortic sympathetic ganglia, and sympathetic trunk ganglia stained intensely for both TrkA and TrkC in the 24-week gestational age fetus with lesser but identifiable immunoreactivity at earlier ages (Figure 3). Mature neuronal cells with pale nuclei and generous cytoplasm stained most intensely. These cells exist in

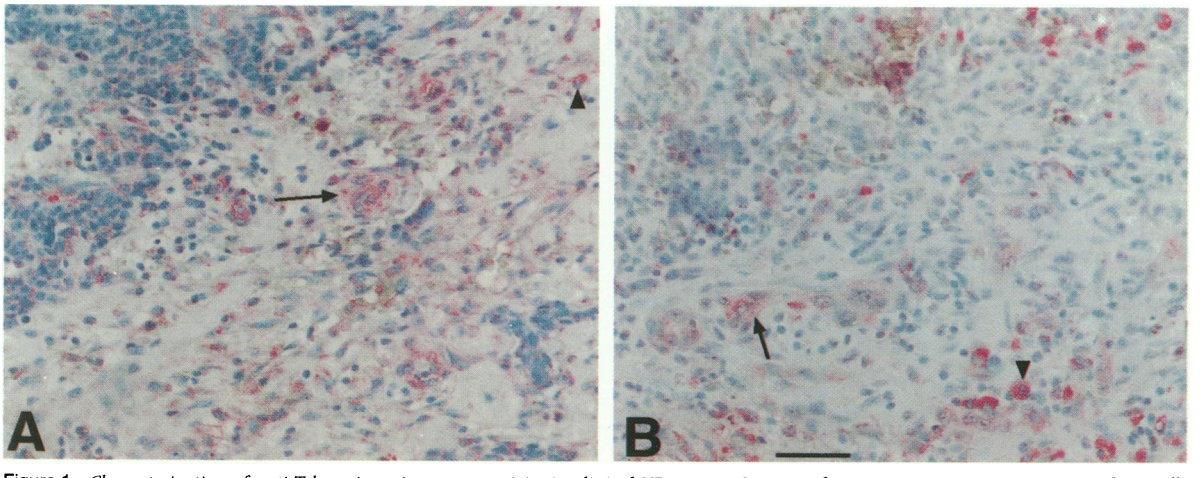

Figure 1. *Characterization of anti-Trk antisera immunoreactivity in clinical NB tumors. Sections of a stage II NB tumor immunocytochemically stained with anti-TrkA (763) antiserum in* (**A**) *and anti-TrkAin antiserum in* (**B**). *Immunoreactive positive cells are indicated by red cytoplasmic staining. Intense positivity detected with both antisera in cytoplasmic-rich, differentiated tumor cells (arrowheads). Differentiated tumor cells forming pseudorosettes are also immunoreactively positive with both antisera (arrows). Less well differentiated groups of tumor cells with dense nuclei and scant cytoplasm lack or show very little Trk immunoreactivity. Scale bar, 200 μm.*

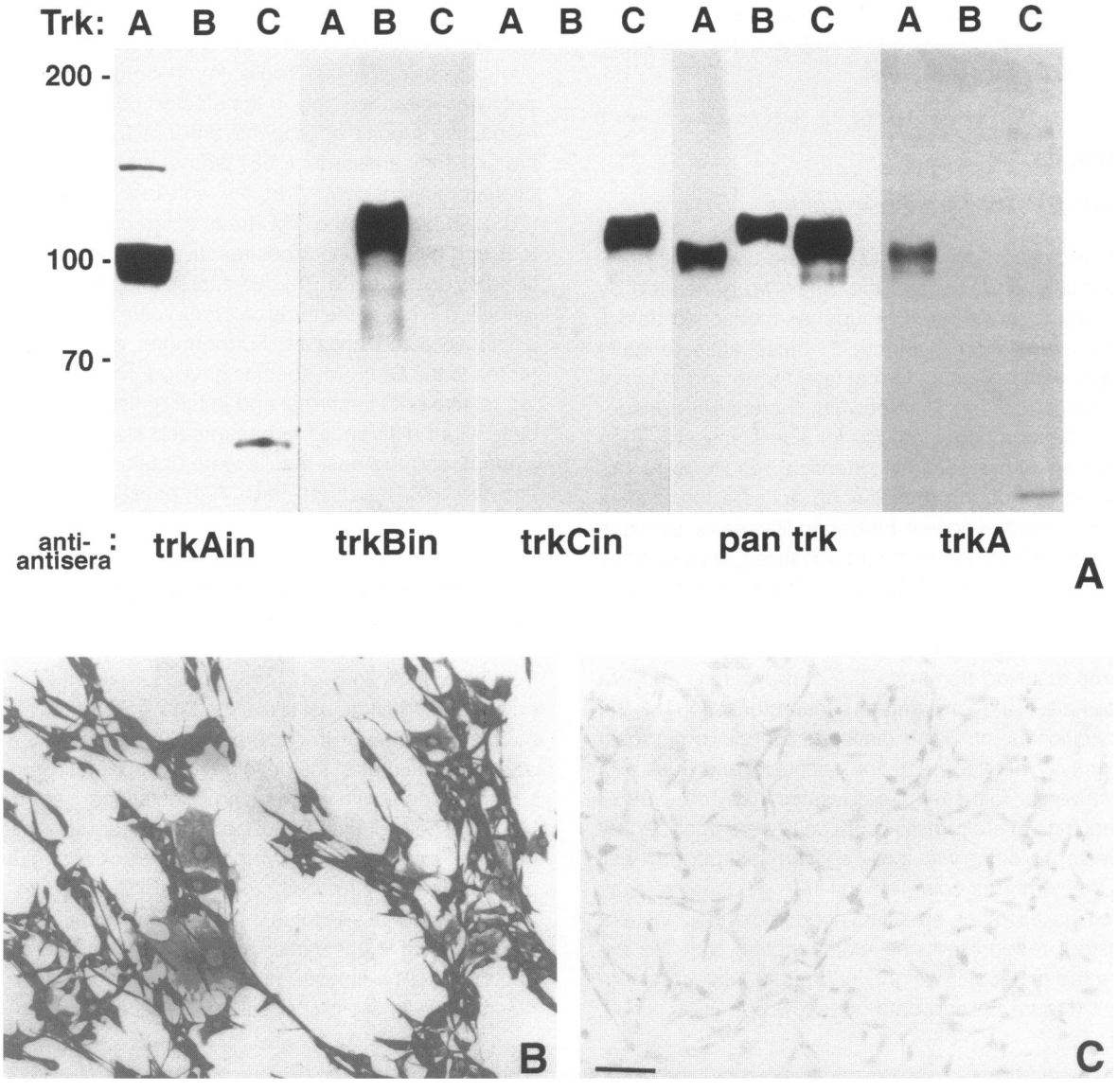

Figure 2. *Characterization of anti-Trk antisera.* **A:** *Western blot analysis of cell lysates of Sf9 cells overexpressing either TrkA (A), TrkB (B), or TrkC (C) was performed with specific anti-Trk antisera as probes. Shown are five identical Western blots prepared from the indicated Trk-overexpressing cultures shown at top. Molecular weight markers in kilodaltons are indicated at left. Note single band at appropriate molecular weight detected in each specific Trk clone by corresponding antisera. No crossover recognition of anti-Trk antisera to other Trk species was evident.* **B:** *Immunocytochemistry of NIH-3T3 TrkA-overexpressing cells stained with anti-TrkA (763) antiserum. Dark cytoplasmic staining of cells indicates immunopositivity.* **C:** *Immunocytochemistry of NIH-3T3 TrkA-overexpressing cells stained with anti-TrkC (798) antiserum. No immunoreactivity was detected. Scale bar, 50 μm.*

greater abundance at later gestational ages. Poorly differentiated sympathetic neuronal cells stained less intensely for TrkA and TrkC, with diminished immunoreactivity at earlier gestational ages. In these tissues, TrkB immunoreactivity ranged from nonexistent to barely discernible (Table 1). Both chromaffin cells and primitive neuronal cells within the adrenal medulla revealed detectable but less intense immunoreactivity with TrkA and TrkC. Paraganglia cells of the organ of Zuckerkandl exhibited intense anti-TrkB antiserum immunoreactivity only at later gestational ages (Table 1). Similarly, only trace staining of mature peripheral nerve trunks was apparent for TrkA and TrkB. Immunoreactivity of the truncated form of TrkB was similar to that of TrkB with the exception of enhanced smooth muscle reactivity. Immunoreactivity with pan-Trk antibody confirmed staining as the sum of all three Trk immunoreactivities. Other fetal tissues also stained positively with anti-Trk antisera, specifically liver and peripheral macrophages with TrkB and pancreatic islet cells with TrkA and TrkB antisera (data not shown).

Table 1. *Neurotrophin Receptor Expression in Human Fetal Peripheral Nervous System*

	Paraganglia	DR ganglia	Sympathetic ganglia	Nerve trunks	Adr medulla	Adr neuronal
TrkA						
8–9 weeks		Trace	Trace	Trace		
13–15 weeks	+	+	+	Trace	Trace	+
21–24 weeks	++	++	++	Trace	Trace	+
TrkB						
8–9 weeks		–	–	–		
13–15 weeks	Trace	–	–	Trace	–	–
21–24 weeks	++	Trace	Trace	Trace	Trace	–
TrkC						
8–9 weeks		Trace	–	–		
13–15 weeks	+	+	+	–	–	+
21–24 weeks	++	++	++	–	+	+

Expression of TrkA, TrkB, and TrkC in 8–9, 13–15, and 21–24-week gestational age human fetuses confined to paraganglia, dorsal root ganglia (DR ganglia), sympathetic ganglia, peripheral nerve trunks, adrenal medullary chromaffin cells (Adr medulla), and primitive neuronal cells of the adrenal medulla (Adr neuronal). (–) indicates absent immunoreactivity; (+), immunoreactive positivity; (++), intense immunoreactivity; and Trace, trace immunoreactivity.

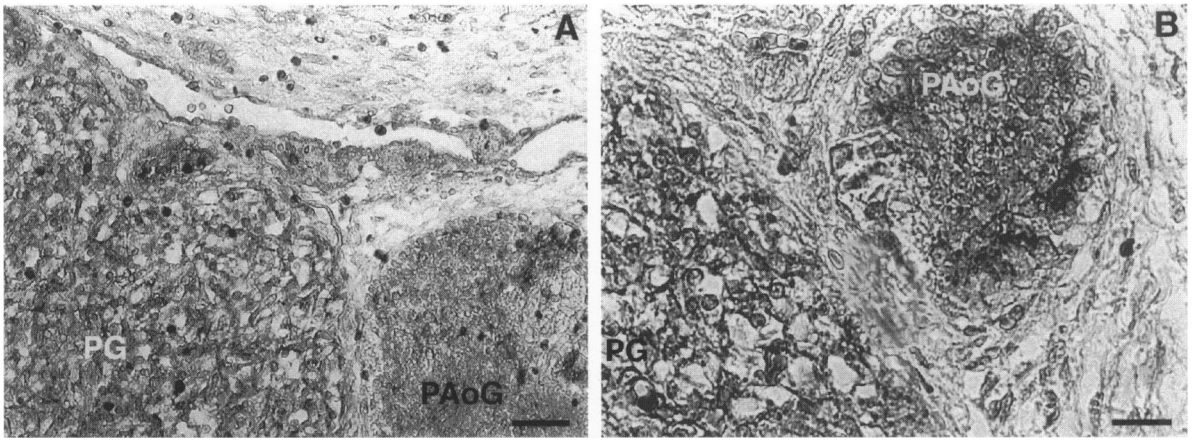

Figure 3. *TrkA immunoreactivity in human fetal tissues.* **A:** *Abdominal cross section through pre-aortic region of 25-week gestational age fetus. Immunoreactivity (763 antiserum) represented by cytoplasmic staining of pre-aortic sympathetic ganglia (PAoG), and paraganglia (PG).* **B:** *High power photomicrograph of pre-aortic area of 25-week gestational age fetus. Immunoreactivity is most evident in sympathetic neuroblasts with abundant cytoplasm and pale nuclei. Staining of paraganglia also evident. Counterstaining was not performed. Scale bar, 200 μm* (**A**) *25 μm* (**B**).

TrkA Expression in Neuroblastoma

All tumors of the 29 infants and children with NB, without exception, stained positively for TrkA to varying degrees (Figure 4, A and B). Staining was both membranous and cytoplasmic, without evidence of nuclear staining, and specific, as revealed by blocking the immunoreactivity by antisera preincubation with the corresponding immunization peptide (data not shown). Equivalent TrkA staining was observed in identical snap-frozen specimens, fixed in acetone, compared with formalin-fixed paraffin-embedded specimens. Only neuroblastoma cells proper stained positively; the stroma and/or fibrous aspects of the tissue did not stain with TrkA antisera. All NB specimens exhibited heterogeneity with respect to degree of cellular differentiation. Differentiated neuroblasts with a low nuclear to cytoplasmic ratio and pale nuclei stained most intensely for TrkA (Figures 1 and 4, A and B). Less mature cells, with densely hematoxylin-stained nuclei and little cytoplasm, stained less intensely. In tumors without a well developed fibrovascular supportive stroma, the anti-TrkA antiserum stained neuroblastoma cells in a random, scattered pattern. In two tested cases with N-*myc* amplification, TrkA immunoreactivity was apparent in both tumor specimens. TrkA expression in the morphologically more mature cells of ganglioneuroblastoma and ganglioneuroma was similar to that of NB, with staining most apparent in mature, differentiated neuronal cells (Figure 5A).

TrkB Expression in Neuroblastoma

Expression of TrkB protein in our selection of NB was detected within the supportive fibrovascular stromal elements of the tumor in a scattered pattern (Figure 4C). Immunoreactivity with an antiserum directed towards the truncated form of TrkB was not appreciably different from

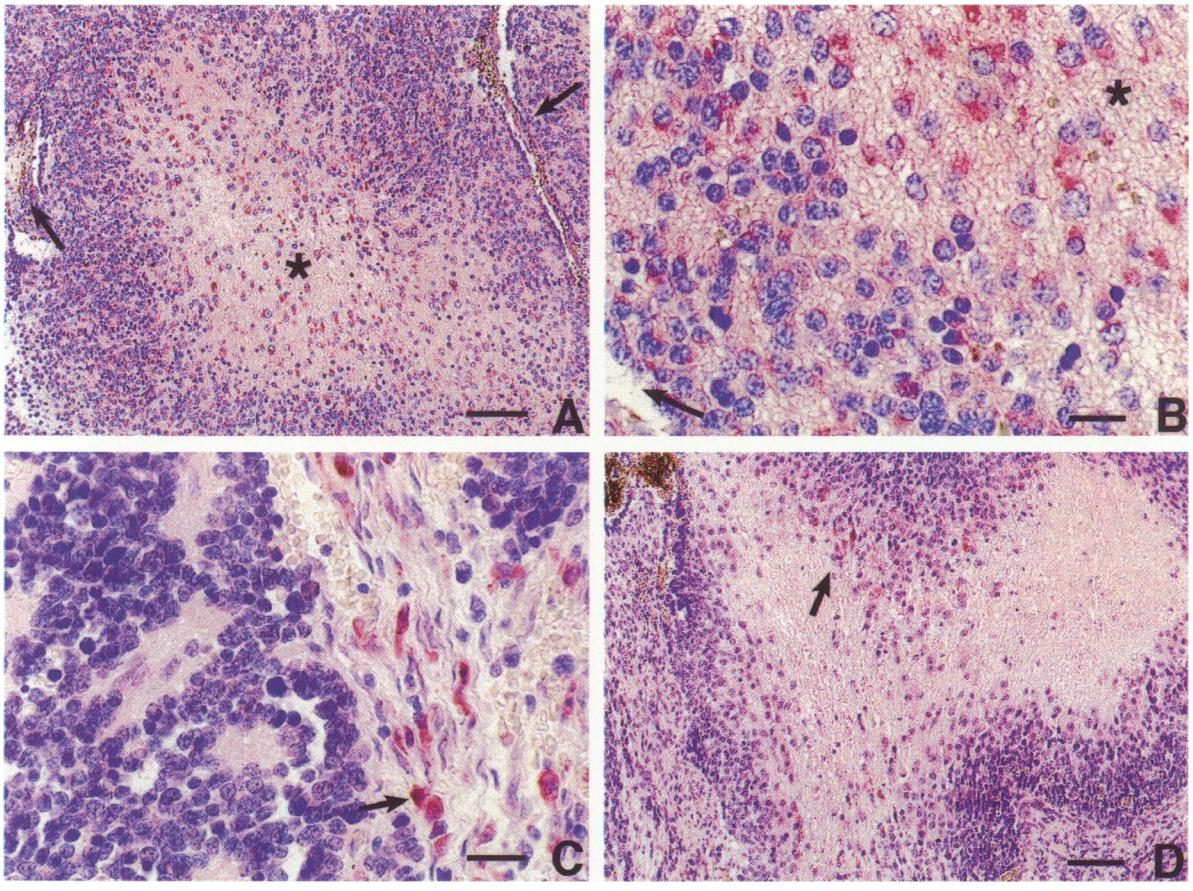

Figure 4. *Trk immunoreactivity in NB.* **A**: *Photomicrograph of an anti-TrkA-stained stage II NB. Note thin, well developed fibrovascular stroma (arrows), zone of malignant neuroblasts, and central zone of cellular remnants (*). Red anti-TrkA (763 antiserum) immunoreactivity is most intense in differentiated neuroblasts lying adjacent to central zone.* **B**: *High power photomicrograph of anti-TrkA-stained specimen* (**A**), *revealing minimal immunoreactivity in neuroblasts adjacent to perilobular fibrovascular stroma (arrow), with intense immunoreactivity in differentiated neuroblasts with pale nuclei and abundant cytoplasm adjacent to central zone (*).* **C**: *Photomicrograph of stage II NB stained with anti-TrkB (794) antiserum. Note intense red immunoreactivity in spindle cells of fibrovascular stroma (arrow) and absent immunoreactivity in primitive neuroblasts proper (left).* **D**: *Photomicrograph of stage IVS NB stained for TrkC (798 antiserum). Note centralized dumbbell-shaped zone of cellular remnant adjacent to differentiated immunoreactive neuroblasts (arrow), bounded by peripheral tumor stroma. Scale bar, 200 μm* (**A** *and* **D**) *35 μm* (**B** *and* **C**).

that of the anti-TrkB antiserum. Immunoreactive positive cells within fibrovascular stroma morphologically resembled macrophages, fibroblasts, or Schwann cells. TrkB immunoreactivity mirrored that of the Schwann cell marker S100 (not shown). In general, fibrovascular stroma-rich tumors displayed greater TrkB immunoreactivity than stroma-poor tumors, with the majority of immunoreactivity within the non-tumor-cell compartments of the tissue. Immunoreactivity of neuroblastic tumor cells proper was confirmed in only one fatal N-*myc*-amplified stage IV tumor. No staining correlation with respect to extent of cellular differentiation was identified with anti-TrkB antisera.

TrkC Expression in Neuroblastoma

As with TrkA immunoreactivity, all tumors examined displayed TrkC immunoreactivity to varying degrees. Staining was specific and appeared to be both cytoplasmic and membranous. (Figure 4D) Neuroblastic cells stained most intensely, with minimal evidence of staining within stromal supportive elements. As in the TrkA stainings, neuroblasts with a low nuclear to cytoplasmic ratio and pale nuclei, more highly differentiated tumor cells, stained most intensely. (Figure 5B) Morphologically less mature neuroblasts displayed significantly less immunoreactivity. In tissues with a disorganized arrangement lacking a well developed fibrovascular stroma, immunoreactive cells were randomly scattered throughout. As with TrkA, ganglioneuroblastoma and ganglioneuroma TrkC immunoreactivity was most intense in more mature, differentiated neuronal cells.

Quantitation of Trk Expression

Statistically significantly increased TrkA expression was apparent in tumor specimens of patients with im-

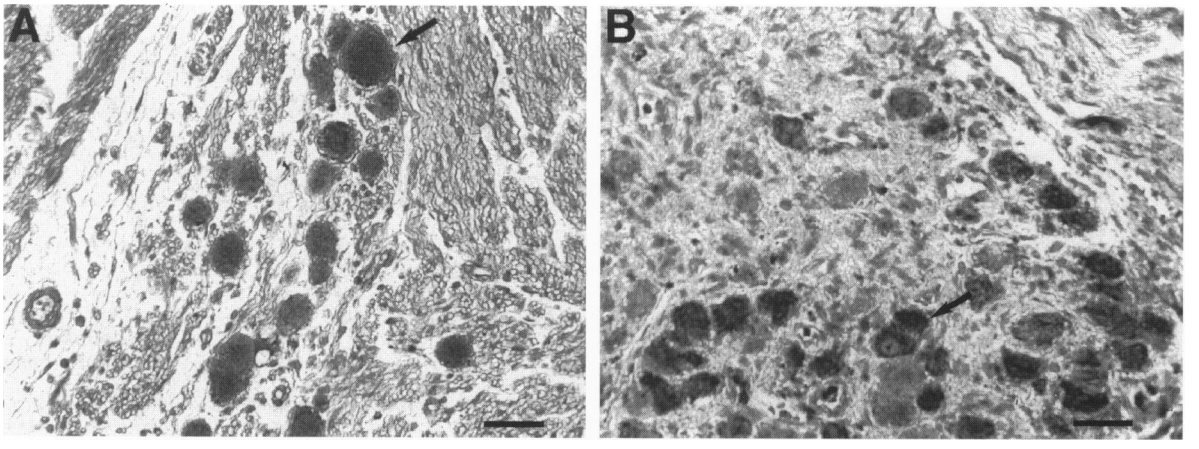

Figure 5. *Trk immunoreactivity correlation with maturation.* **A:** *Anti-TrkA (763 antiserum)-stained ganglioneurona tumor. Note intense cytoplasmic immunoreactivity of large cytoplasmic-rich, mature neuronal cells (arrow), with absent staining of surrounding supportive elements.* **B:** *Anti-TrkC (798 antiserum)-stained photomicrograph of stage IV NB. Immunoreactivity is most evident in ganglia-like, mature, differentiated neuroblasts with abundant cytoplasm and pale nuclei (arrow). Immature neuroblastic cells and supportive elements are TrkC negative. Counterstained with hematoxylin; scale bar, 70 μm* (**A**), *35 μm* (**B**).

proved outcome (2.56 ± 0.80 for survivors *versus* 1.91 ± 0.29 for nonsurvivors; $P = 0.026$), and with favorable tumor stage (3.0 ± 0.91 for stages I, II, and IVS *versus* 1.88 ± 0.48 for Stages III and IV; $P = 0.0027$). The semiquantitative analysis of TrkA expression is represented in Figure 6. A similar trend was seen for TrkC immunoreactivity, but statistical significance was not achieved ($P = 0.08$). No statistical differences were identified for TrkB immunostaining stratified according to outcome or tumor stage ($P > 0.05$).

NB Lobule: TrkA and TrkC Expression and Maturation

In NB tissue with more organized histology, primarily low stage tumors, a specific pattern of TrkA and TrkC immunoreactivity was evident. In tumor sections with a lobular or acinar arrangement of tumor cells, TrkA and TrkC expression was least evident in poorly differentiated neuroblasts adjacent to the perilobular stroma, capillaries, and blood vessels (Figure 4). These cells typically displayed an increased nuclear to cytoplasmic ratio with dense nuclei and scant cytoplasm. TrkA and TrkC expression progressively increased in cells nearing the center of the lobule structure, near that of the central cellular remnant zone (Figure 7). Cells about this cellular remnant zone have pyknotic and condensed nuclei, with fragmented DNA as demonstrated by the TUNEL technique, suggestive of an apoptotic cellular death process (Figure 8). Cells expressing TrkA and TrkC displayed pale nuclei and more abundant cytoplasm than those small, undifferentiated cells lying adjacent to the stroma and capillaries. Similarly, NSE and chromogranin A&B immunoreactivity, as well as hematoxylin and eosin morphology, confirmed increasing cellular maturation in neuroblasts nearing the lobule center (not shown).

Discussion

Our investigation included an examination of human fetal tissue in conjunction with that of NB because of the known and presumed prenatal generation of NB, the prognostic dichotomy of NB relative to age of diagnosis, a lack of understanding of NB progenitor cell(s), and the known existence of spontaneous regression in NB. Furthermore, the potential importance of apoptosis in both NB and within the developing sympathetic nervous system lead us to assume that fetal tissue characteristics are important in evaluating the generation of NB.

Trk immunoreactivity was evident within the developing human sympathetic nervous system. Paraganglia, pre-aortic ganglia, and sympathetic trunk ganglia, the presumptive progenitors of neuroblastoma, stained intensely with TrkA and TrkC at later gestational ages. In these structures, immunoreactivity to all Trk antibodies increased with increasing gestational age. The adrenal medulla, consisting of both chromaffin and primitive sympathetic neuronal cells at the developmental stages studied, revealed detectable but less intense TrkA and TrkC immunoreactivity. Sensory dorsal root ganglia stained with all Trk antibodies but only minimally so for TrkB. Interestingly, at the latest gestational ages, intense TrkB immunoreactivity was localized only to paraganglia.

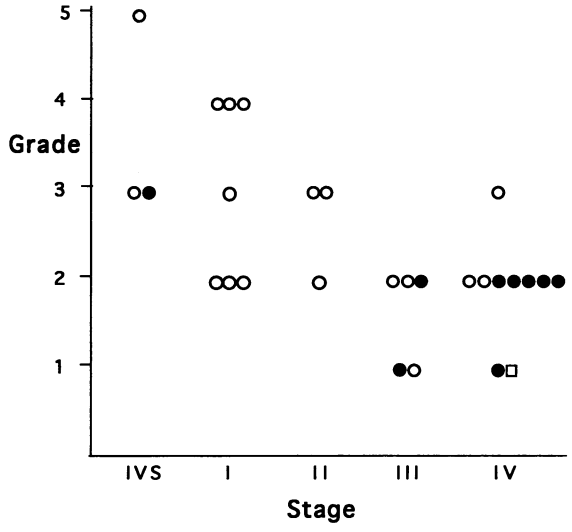

Figure 6. *Semiquantitative grading of TrkA expression in NB.* **A:** *Graphic representation of TrkA immunoreactivity grade (ordinate) in NB stratified according to tumor stage (abscissa). Open circles (○) denote long-term survivors, filled circles (●) denote nonsurvivors, and open square (□) denotes alive with active disease. The single death in the stage IVS group occurred as a result of neuroendocrine crisis rather than tumor burden. Note trend of diminishing staining grade with tumor stages of worsened prognosis. Statistical comparisons between survivors and nonsurvivors by Student's* t-test *reveals statistically significant increased TrkA staining in survivors* versus *nonsurvivors* ($P = 0.026$).

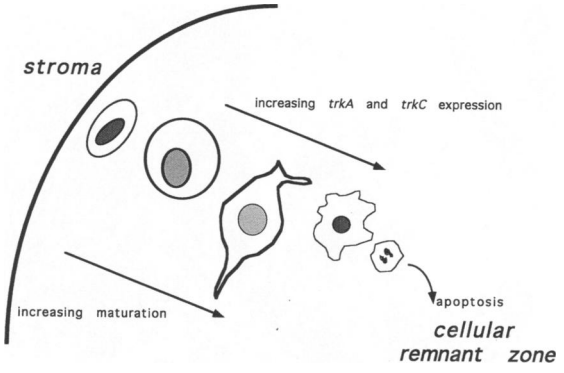

Figure 7. *Neuroblastoma lobule: maturation and Trk expression. Representation of NB tumor lobule as described in text. Low TrkA and TrkC expression exists in neuroblasts adjacent to tumor stroma. An increase in TrkA and TrkC expression occurs with increasing maturation as neuroblasts locate toward central cellular remnant zone. Cellular death by apoptosis occurs at the center of the lobule structure.*

With the presumptive sites of NB being that as mentioned above, altered TrkA and TrkC signaling in these tissue types could influence malignant cell growth.

In NB tissues, immunoreactivity of tumor cells proper was most evident with TrkA and TrkC antisera. In accordance with *trkA* expression previously reported at the mRNA level,[20,25–27] we similarly found increased TrkA protein expression in favorable outcome, low stage tumors. Neither TrkB nor TrkC expression grade conclusively correlated with prognosis or tumor stage. Therefore, the possibility that NGF via TrkA responsiveness directs NB tumor growth is feasible. Others have shown that N-*myc* amplification, extent of neuronal differentiation, and the mitosis karyorrhexis index correlate with tumor stage and prognosis.[14,19–21] Previously, correlations of the degree of apoptosis, maturation, and expression of the death suppressor proto-oncogene *bcl-2* with favorable outcome have been reported.[24,34] The current study suggests that TrkA and TrkC expression also vary with neuronal differentiation. Within a given tumor, differential degrees of cellular maturation often exist. In those tumor cells of immature morphology, both TrkA and TrkC expression was low. We found increased expression of both TrkA and TrkC in more mature tumor cells, as evidenced by morphology, and NSE and chromogranin A&B immunoreactivity. This spatial arrangement of maturation and Trk expression is most apparent in lower stage tumors (stages I, II, and IVS) with improved prognosis in contrast to those with worsened prognosis (stages III and IV).

Recently, expression of *trkB* mRNA in homogenates of poor prognosis high stage NB has been reported, with truncated *trkB* expression found primarily in benign ganglioneuroblastoma and ganglioneuroma.[35] Similarly, retinoic acid-induced NB cell cultures also express full length and truncated *trkB*.[28] We report no prognostic dependency of TrkB expression in our limited NB material; however, TrkB expression was identified in non-neuroblastic cells lying within fibrovascular stroma and in neuroblastic cells of a single N-*myc*-amplified tumor. This finding that TrkB immunoreactivity exists within fibrovascular stroma cells may indicate that tumor stromal cells and hence tumor angiogenesis through paracrine interactions are dependent upon this form of neurotrophic support. Our limited number of N-*myc* amplified tumors available make conclusions regarding TrkB expression in this tumor form irrelevant; however, our single case with neuroblastic TrkB positivity is consistent with the reported TrkB expression in some NB cell lines and tumors with N-*myc* amplification.[27,34]

The maturational spatial arrangement of Trk expression that we describe may provide an explanation for the biological activity of these tumors. In NB tumors of lesser stage, neuroblastic cells residing adjacent to the fibrovascular stroma are poorly differentiated, lacking Trk expression. As these primitive cells presumably locate away from this stroma, they mature morphologically with subsequent increased chromogranin A&B and NSE expression. We also find

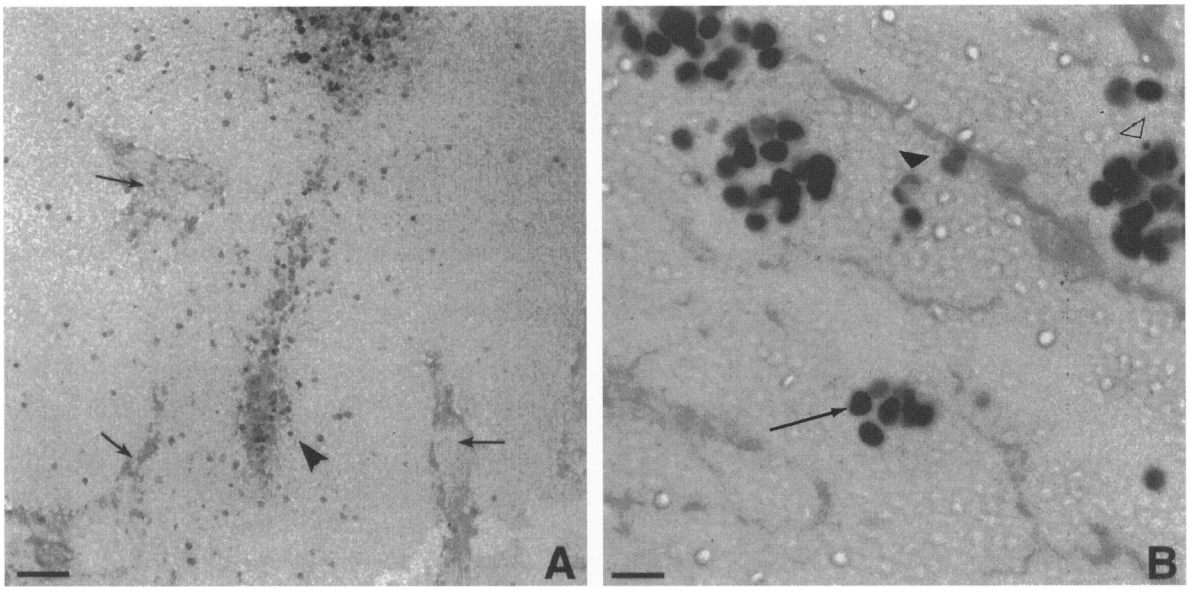

Figure 8. *Detection of apoptosis in NB.* **A:** *Photomicrograph of stage II NB stained by TUNEL technique. Fibrovascular stroma margins of a characteristic tumor lobule demarcated by black arrows. Lobule center of cellular remnants reveals adjacent scattered nuclear cellular staining (arrowhead). Minimal nuclear staining is apparent in the majority of neuroblasts and about tumor stroma.* **B:** *High power view of* (**A**) *of a large group of apoptotic cells adjacent to cellular remnant zone. Note intense, nuclear specific staining of apoptotic neuroblasts (arrow), the majority of which reveal nuclear condensation. Cells with fragmented nuclei (black arrowhead) as well as apoptotic bodies (open arrowhead) are also TUNEL positive. Scale bar, 250 μm* (A), *25 μm* (B).

that other tumor cells located adjacent to these differentiated cells are pyknotic, with condensed nuclei and fragmented DNA suggestive of apoptotic cell death. With differentiation *in situ*, TrkA and TrkC expression increase, or the corollary, as Trk expression increases, the cells undergo differentiation. This might suggest that, as NB cells acquire increasing degrees of differentiation, they develop an increased capability to respond to neurotrophins by increased receptor expression. Whether this occurs is uncertain; however, one study suggests that tumor cells acquired from good prognosis, TrkA-expressing tumors, do differentiate in response to NGF, with NGF deprivation resulting in cellular death.[20] It is not known whether the same is true of tumors that express TrkB or TrkC. This might suggest that a deficiency in a circulation-derived substance, such as a metabolic substrate or growth factor, or relative ischemia may regulate Trk expression and incite cellular differentiation. Tumors with high Trk expression do show higher levels of the differentiation markers NSE, synaptophysin, and neuronal c-*src* protein[36] and, therefore, increased expression of the neurotrophin receptors in good prognosis tumors may merely be a reflection of a more differentiated and therefore less aggressive state.

An obvious shortcoming of this study is the inability to acquire tumor specimens before chemotherapeutic treatment in all patients with high stage tumors. Others have indicated that various chemotherapeutics exert their effect by induction of apoptosis and cellular death mechanisms,[37] suggesting that these agents might act through a type of cellular maturation pathway as well. It is conceivable that Trk expression alterations could occur in those tumors acquired after chemotherapeutic treatment; however, the histological features of the neuroblastoma lobule we describe should remain unaltered as this pattern is most apparent in nontreated low stage tumors and persists in tumor specimens obtained both before and after treatment.

The question regarding Trk expression and neurotrophin responsiveness in NB has become an important one. It is known that most NB cell lines are trophic and tropic unresponsive to NGF.[19,38] The majority of these cell lines, however, are derived from high stage, aggressive, N-*myc*-amplified tumors.[19] A variety of NB cell cultures nevertheless do express *trk* and the low affinity NGF receptor p75 in lieu of their NGF unresponsiveness. In fact, when cultured NB cells are induced to undergo differentiation by a variety of means, Trk expression increases but with continued NGF unresponsiveness.[39] NB cell lines exogenously transfected with *trk* do become NGF responsive with resultant growth and differentiation, even though these cells express endogenous *trk* before transfec-

tion. High stage NB may be NGF or neurotrophin unresponsive either by a gene dose-related phenomenon whereby cellular receptivity to neurotrophins is diminished or possibly by structural defects in endogenous $p75^{NGFR}$ or Trk.

We postulate that in low stage NB, TrkA or TrkC responsiveness may play a role in persistent tumor growth or regression. In contrast, higher stage tumors maintain a less effective mechanism of neurotrophin reactivity, at least with respect to TrkA and the neurotrophin NGF. Others have described coexistence of both the neurotrophins and their receptors in the developing nervous system and confirmed responsiveness and expression of the neurotrophins within the same tissue.[9,13] Investigations to determine whether neurotrophin activity exists within the neuroblastoma tissue or whether neurotrophins are blood borne or endogenously produced by the tumor cells or stroma have not been successful thus far. Neurotrophic influences might also be important in the cellular maturation and/or subsequent self-destruction of NB; ie, a lack of neurotrophin support may result in cellular death. Ultimately, the potential to potentiate neurotrophic influences in NB tumors with consequent induction of differentiation may also prove to encourage cellular death via apoptosis.

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